REMARKS

Claims 94 and 108-109, and 117-120 are currently pending in the application. No new matter has been introduced by virtue of the within response.

Claims 94, 108-109, and 117-120 were rejected under 35 U.S.C.§103(a) as being allegedly unpatentable over Sananaka or Masahiro or Zhang or or Tamiko for the reasons of record.

The Office action alleges that the data presented by the Declaration submitted on February 28, 2005 did not convicingly demonstrrate unexpected results for the claimed invention.

Applicants respectfully disagree.

The pending final office action has maintained the §103(a) rejections – over Sakanaka, Masahiro, Zhang and Tamiko. In particular, the position was taken that the amounts recited in the claims of the present application would have been obvious in view of the cited art.

In particular, the position was taken that the data of the Declaration and the Zhang document were contradictory such that the declaration did not support a holding of unexpected results.

Thus, Examiner averred in the pending Final Office Action that "Zhang, et al. clearly teach that 'Rb_1 10-40 mg/kg iv before and after reduced the IS by 20-49% and 12-35% respectively' (IS = infarct size) (p. 46, second paragraph)." Zhang et al. further comment that 'Rb_1 that the same procedure resulted in a 22% decrease in occlusion for 10 mg Rb_1/kg/day and a 50% decrease in occlusion for 40 Rb_1 mg/kg/day (see Table 1, p. 46)'." The Examiner concludes that there has been an error in the protocol of the experiment of the Declaration because there is a contradiction with the experimental result shown in Zhang et al. However, Applicants respectfully submit that the conclusions reached by the Examiner are not proper due to improper comparisons

between the data obtained from different animal models. That is, the Zhang data was obtained by different ischemia models and different modes of administering ginsenoside Rb1.

As the Zhang publication is understood, the permanent middle cerebral artery occlusion model (Model 2 of the following table) was carried out by inserting a glass fiber into the middle cerebral artery of the subject rat and the transient middle cerebral artery occlusion model (Model 1) was carried out by inserting the glass fiber into the middle cerebral artery for two hours followed by removal of the glass fiber. See, page 45 of the Zhang publication in the "Materials and Methods" section under the heading "Rat model."

Zhang recites that ginsenoside Rb1 was administered either **before** ischemia (e.g., occlusion of the middle cerebral artery with a glass fiber) or **immediately following** ischemia (e.g., removal of the glass fiber in the transient middle cerebral artery occlusion experiment). See, page 45, right column, lines 8-9 of the Zhang publication. In contrast, Zhang only administered ginsenoside Rb1 to Model 2 rats receiving permanent middle cerebral artery occlusion **before** insertion of the glass fiber. See, page 45, right column, line 7 of the Zhang publication. Thus, none of the Zhang experiments teach or suggest methods of treating ischemia after a two-hour post delay between occluding the cerebral artery and administering the dose of ginsenoside Rb-1.

In contrast, the experimental data provided in the Declaration filed February 28, 2005 was generated in a rat model (Model 3) in which the "cortical branch of the left middle cerebral artery (MCA) of each animal was coagulated and cut." See, page 2, lines 6-8 of the Declaration filed February 28, 2005. Various Ginsenoside Rb1 doses were then administered intravenously two hours **after** permanent MCA occlusion. See, page 2, lines 8-11 of the Declaration filed February 28, 2005. That is, doses of 10 mg/kg/day, 40 mg/kg/day, 6 μg/kg/day and 60 μg/kg/day were administered to rats two hours post infarction. Thus, the infarct animal model presented in the Declaration uses a more severe infarct injury than the transient or permanent occlusion models presented in Zhang and all experimental data reported in the Declaration used rat

Model 3. Thus, data reported in Zhang using rat Model 1 and rat Model 2 are not directly comparable to the data presented in the declaration, which uses rat Model 3.

In order to particularly point out the differences in the Zhang animal models (Model 1 and Model 2) and the animal model used in the Declaration (Model 3), a chart of the different experimental conditions and results of the infarct treatment protocols recited in Zhang et al and the Declaration filed February 28, 2005 are presented in the table, which follows:

Model Rat	Amount of Rb1 administered iv				
	Rb1 10 mg/kg		Rb1 40 mg/kg		Rb1 20-200 μg/kg
	[e]Adm. before	[f]Adm. after	[e]Adm. before	[f]Adm. after	[f]Adm. after
[a]Model 1	[d]20% reduction	12% reduction	49% reduction	35% reduction	
[b]Model 2	No significant effect	Data not shown	14% reduction	Data not shown	[h]67% reduction
[c]Model 3		No significant effect		[g]No significant effect	50% reduction

(Increasing Severity of Infarct in Model Rat ↓)

a) In Rat Model 1, called ischemia/reperfusion model, cerebral blood flow was occluded only for 2 hours and then resumed. Ginsenoside Rb1 was administered before the occlusion of cerebral blood flow or after the resumption of cerebral blood flow. This model does not represent the pathological condition of cerebral infarction with permanent occlusion of a main cerebral artery including the middle cerebral artery.

b) In Rat Model 2, the middle cerebral artery (MCA) was permanently occluded and ginsenoside Rb1 was administered just before or immediately after the occlusion. Model 2 is widely used for evaluation of cerebral infarction-curing agents.

c) In Rat Model 3, the middle cerebral artery (MCA) was permanently occluded (i.e., the MCA was coagulated and cut) and ginsenoside Rb1 was administered 2 hours after the occlusion. This model is used for analysis of the therapeutic time window of cerebral infarction-curing agents in order to evaluate the clinical efficacy of the agents.

d) "% reduction" in the table means the reduction of cerebral infarct volume.

e) "adm.before" in the table and in the arguments which follow means that ginsenoside Rb1 was administered before ischemia.

f) "adm.after" in the table and in the arguments which follow means that ginsenoside Rb1 was administered after brain ischemia.

g) Although a 7% reduction of cerebral infarct volume was noted on the basis of mean value, the % reduction was not a statistically significant reduction in cerebral infarct volume relative to the control.

h) cerebral infarct area was reduced to 1/2 of control and thus the cerebral infarct volume was reduced to 1/3.

The abbreviations of the above a) to f) are used herein under.

In the Zhang experiments conducted using Rat Model 1, ginsenoside Rb1 10 mg/kg iv or 40 mg/kg iv before and after occlusion showed significant effects as pointed out by the Examiner. Further, in general, administering before ischemic insult usually shows better positive effects than administering after ischemic insult, which is commonly acknowledged by the skilled artisan as pointed out by the Examiner.

Zhang teaches that ginsenoside Rb1 40mg/kg iv exhibits better positive effects in Model 1 and Model 2 experimentswhen compared to that of ginsenoside Rb1 10mg/kg iv. Thus, one of ordinary skill in the art would reasonably expect that higher doses of ginsenoside Rb1 generate superior therapeutic results. Conversely, one of ordinary skill in the art would not have a reasonable expectation of obtaining a desirable result by reducing the dose of ginsenoside Rb1 administered iv to a patients based on the Zhang Model 1 experimental data.

In the Model 2 rat model, which animals are inflicted with a more severe infarct (e.g., permanent insertion of a glass fiber into the middle cerebral artery), Zhang reported that animals administered a ginsenoside Rb1 10mg/kg before occlusion do not exhibit improvement in cerebral infarct volume. Zhang further recites that Model 2 rats administered with a higher does, e.g., ginsenoside Rb1 40mg/kg before occlusion, show slight improvement in cerebral infarct volume, i.e., a 14% reduction in cerebral infarct volume.

However, the Zhang publication does not report administering any dosage of ginsenoside Rb1 after occlusion in the Model 2 rat.

Thus, the rat ischemia model data recited by Zhang suggests to one of ordinary skill in the art that higher dosages provide greater reduction in infarct volume and administration before occlusion provides greater reduction in infarct volume than administration after occlusion. More particularly, the data reported by Zhang teaches

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that a 10 mg/kg dose of ginsenoside Rb1 administered after occlusion of the middle cerebral artery is insufficient to reduce infarct volume and suggests that lower doses of ginsenoside Rb1 administered post-occlusion will be insufficient to reduce infarct volume.

One of ordinary skill in the art would not have a reasonable expectation of obtaining the superior reduction of infarct volume obtained by the claimed invention based on the Zhang reference.

In contrast, in the present invention, ginsenoside Rb1 20-200μg/kg (6-60μg/body) iv administered two hours post occlusion shows great therapeutic effects on Model 2 rats, which results is unexpected in view of the Zhang study. In fact, the lower amounts of ginsenoside Rb1 iv two hours after occlusion reduced the areas of cerebral infarction to 1/2 or less of the control administered with vehicle (physiological saline), which corresponds to the reduction of the cerebral infarct volume to 1/3 - 1/4 of the control. The reduction of cerebral infarct volume is around 67% (please refer to the table).

Further, the present inventors conducted an additional experiment, using Model 3 rats in which ginsenoside Rb1 treatment was administered two hours after the onset of cerebral infarction. This experiment shows that administration of ginsenoside Rb1 20-200μg/kg iv two hours after occlusion reduced the cerebral infarct volume to 1/2 of the control administered with physiological saline alone even 24 hours after the permanent occlusion of the middle cerebral artery.

Although Zhang does not recite administration of ginsenoside Rb1 after permanent occlusion of a cerebral artery, Applicanst administered 10 mg/kg/day and 40 mg/kg/day doses of ginsenoside Rb1 to Model 3 Rats. As shown in the Declaration filed February 28, 2005, no significant effect was observed at the higher ginsenoside doses.

Thus, as discussed *supra*, there is no contradiction or inconsistency between the data presented in Zhang et al., the specification as filed or in the Declaration filed

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February 28, 2005, at least because different rat ischemia models were recitedin Zhang as compared to the specification and Declaration.

The experimental results concerning low doses and low concentrations of ginsenoside Rb1 as discussed above, are more fully discussed in B. Zhang, et al., *Prevention of ischemic neuronal death by intravenous infusion of a ginseng saponin, ginsenoside Rb1, that upregulates Bcl-x$_L$ expression*, J. Cerebral Blood Flow & Metabolism, 2005, pp 1-14. A copy of the publication is attached hereto for the convenience of the Examiner.

Thus, for at least the reasons discussed herein, claim 94 is patentable over Zhang. Claims 108, 109, 117-120 depend from claim 94 and are therefore also patentable over Zhang.

Referring now to Sakanaka and Masahiro, these references recite that crude saponin fraction(s) of ginseng and ginsenoside Rbl when administered by the dosage and administration routine disclosed therein have the effect of preventing brain ischemia.

Sakanaka and Masahiro teach that **elevated doses** of crude saponin fraction(s) of ginseng and ginsenoside Rbl recited by Masahiro and Sakanaka may be effective to cerebrovascular disorder or cerebral infarction by these **elevated dosages**. Moreover, each of Masahiro and Sakanaka teach that with regard to the dosage for the preventive effect of crude saponin fraction(s) of ginseng, 100mg/kg/day is superior to 50mg/kg/day, and with regard to ginsenoside Rb1, 20mg/kg/day is superior to 10mg/kg/day. In short, these references teach that elevated dosages are superior. In that way, the references effectively teach away from the present invention.

Clearly, one skilled in the art would not have been motivated to decrease the dosages below the ranges recited in Sakanaka and Masahiro. The Final Office Action cites MPEP§2144.05 Part II A for the premise that modification of dosage amount is routine experimentation. However, the instant claims provide methods of treatment of or prevention of diseases causing apoptosis or apoptosis-like death of cells by

administration of a doses or dosages of ginseng extracts are adjusted to between 145 pg/kg/day and 1450 μg/kg/day, and those of ginseng components are adjusted to between 1.67 pg/kg/day and 1.67 mg/kg/day. The dose or dosage provided by claim 94 (as amended by the First Amendment After Final Rejection), is at least one order of magnitude lower than those recited in Sakanaka or Masahiro. Moreover, Sakanaka and Masahiro teach that higher dosages provide greater therapeutic effect. Thus, although some modification of dosage may be reasonable, at the time the invention was made, one of ordinary skill in the art would have been directed by the teaching and suggestion of Sakanaka or Masahiro to increase, not decrease, the dosage of red ginseng to a patient susceptible to ischemia.

In contrast, Applicants have surprisingly discovered that mammals suffering from spinal cord injury or cerebral infarction can be treated by administration of crude saponin fraction(s) of ginseng and/or ginsenoside Rbl (which is one of the ginsenoside compositions) at unprecedented low dosage.

Additionally, in support of the arguments stated herein, attention is directed to the enclosed **Rule 132 Declaration of Masahiro Sakanaka**. The Rule 132 Declaration details certain experiments conducted to compare the therapeutic effect of ginsenoside Rb1 administered in low-dosage form according to the present invention with that of ginsenoside Rb1 administered in high dosage form, e.g., as in the cited art. The evidence provided in the Rule 132 Declaration shows the that the low-dosage ginsenoside Rb1 is highly superior to the high-dosage ginsenoside Rb1 in terms of the clinically applicable therapeutic time window. Such an effect is very surprising and non-obvious in view of the art cited. The evidence provided rebuts any case of alleged obviousness that may be contended.

As discussed *supra*, the Declaration does not contradict the data of Zhang. Thus, the Declaration provides evidence of unexpected results for the claimed invention.

It is respectfully submitted, therefore, that claim 94 is patentable and non-obvious over Sakanaka, Masahiro, or any combination thereof. Claims 108, 109, and

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117-120 depend from claim 94 and are therefore also patentable over Sakanaka, Masahiro, or any combination thereof.

Claims 94, 108-109, and 115-120 stand rejected under 35 U.S.C. §103(a) over Tamiko.

Claim 94, as amended, is patentable over Tamiko. Claims 108, 109, and 117-120 depend from claim 94 and are therefore also patentable over Tamiko.

The claims of the present application provide methods of treating a mammal suffering from or susceptible to diseases causing apoptosis or apoptosis-like death of cells, except for treatment of immune deficiency, which comprises administering to the mammal a composition comprising ginseng extracts, or ginseng components, its metabolites or salts thereof. Thus, the claims do not provide methods of treatment comprising administration of ginseng. Therefore claims 94, 108, 109, 117-120 are patentable over Tamiko.

The rejections are therefore properly withdrawn. For instance, it is well-known that to establish a *prima facie* case of obviousness, three basic criteria must be met: (1) there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine reference teachings; (2) there must be a reasonable expectation of success; and (3) the prior art reference(s) must teach or suggest all the claim limitations. The teaching or suggestion to make the claimed combination and the reasonable expectation of success must both be found in the prior art, and not based on applicant's disclosure. *In re Vaeck*, 947 F.2d 488, 20 USPQ2d 1438 (Fed. Cir. 1991). See MPEP § 2143.

There is no suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the cited references to make the claimed invention, nor is there a reasonable expectation of success.

In view thereof, reconsideration and withdrawal of the §103 rejections are requested.

In view of the above amendment, applicant believes the pending application is in condition for allowance.

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Respectfully submitted,

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Prevention of ischemic neuronal death by intravenous infusion of a ginseng saponin, ginsenoside Rb₁, that upregulates Bcl-x$_L$ expression

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Almost all agents that exhibit neuroprotection when administered into the cerebral ventricles are ineffective or much less effective in rescuing damaged neurons when infused into the blood stream. Search for an intravenously infusible drug with a potent neuroprotective action is essential for the treatment of millions of patients suffering from acute brain diseases. Here, we report that postischemic intravenous infusion of a ginseng saponin, ginsenoside Rb₁ (gRb₁) ($C_{54}H_{92}O_{23}$, molecular weight 1109.46) to stroke-prone spontaneously hypertensive rats with permanent occlusion of the middle cerebral artery distal to the striate branches significantly ameliorated ischemia-induced place navigation disability and caused an approximately 50% decrease in the volume of the cortical infarct lesion in comparison with vehicle-infused ischemic controls. In subsequent studies that focused on gRb₁-induced expression of gene products responsible for neuronal death or survival, we showed that gRb₁ stimulated the expression of the mitochondrion-associated antiapoptotic factor Bcl-x$_L$ *in vitro* and *in vivo*. Moreover, we revealed that a Stat5 responsive element in the bcl-x promoter became active in response to gRb₁ treatment. Ginsenoside Rb₁ appears to be a promising agent not only for the treatment of cerebral stroke, but also for the treatment of other diseases involving activation of mitochondrial cell death signaling.
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Keywords: Bcl-x$_L$; cerebral ischemia; ginsenoside Rb₁; Stat5

Introduction

Approximately 4 million people die of cerebral stroke, and more people with stroke become handicapped every year in the world, possibly because of the lack of intravenously infusible drugs with a

potent neuroprotective action. Almost all agents that exhibit neuroprotection when administered into the cerebral ventricles are ineffective or much less effective in rescuing damaged neurons when infused into the blood stream (Wu and Pardridge, 1999). This is because such neuroprotective agents do not pass through the blood brain barrier and/or they are likely to be degraded into nonbioactive fragments in the blood stream, mostly by the actions of proteases.

Over the past several decades, biomedical researchers have been making desperate efforts, without any great success, to develop intravenously infusible neuroprotective agents, with great emphasis on glutamate receptor antagonists like MK-801. However, most glutamate receptor antagonists, even though alleviating ischemic neuronal damage, frequently cause serious adverse effects in humans by modulating neural transmission in an adverse

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manner, and might not be applicable for clinical use. A search for intravenously infusible neuroprotective agents that act selectively on damaged brain tissue without affecting neural transmission appears to be essential for the treatment of cerebral stroke. We speculate that development of an intravenously infusible agent with a potent neuroprotective action would be absolutely of value from the clinical point of view, because such an effective agent would greatly contribute to elucidation of the molecular mechanisms underlying the brain diseases for which the agent is applied, and would facilitate the development of other innovative treatment protocols or drugs over the next 5 to 10 years or beyond.

Ginseng root (Panax ginseng CA MEYER) has been prescribed to patients for thousands of years in Asian countries, without apparent adverse effects. Ginseng root consists of two major ingredients: crude ginseng saponin and crude ginseng non-saponin fractions. To date, more than 20 saponins have been isolated from ginseng root and identified chemically. They can be classified into three major groups according to their chemical structure: protopanaxadiol, protopanaxatriol and oleanolic acid saponins, of which ginsenoside Rb_1 (gRb_1), ginsenoside Rg_1 and ginsenoside R_0 are representative substances, respectively (Shibata et al, 1994).

In the present study, we first showed that postischemic intravenous infusion of gRb_1 ameliorated cerebrocortical infarct size, place navigation disability and secondary thalamic degeneration in spontaneously hypertensive rats-stroke prone (SHR-SP), with permanent occlusion of the unilateral middle cerebral artery (MCA) distal to the striate branches. In subsequent in vitro and in vivo experiments that focused on gRb_1-induced expression of gene products responsible for neuronal death or survival, we showed that gRb_1 stimulated neuronal $Bcl-x_L$ protein expression and prevented neuronal apoptosis at concentrations of 1 to 100 fg/ml in vitro, and that postischemic intravenous infusion of gRb_1 in MCA-occluded SHR-SP induced $Bcl-x_L$ expression.

Materials and methods

All experimental procedures were conducted according to the Guide for Animal Experimentation at Ehime University School of Medicine. Animals were housed in an animal room with a temperature range of 21 to 23°C and a 12-h light/dark cycle (light on: 0700 to 1900 h). Animals were allowed access to food and water ad libitum until the day of the experiment.

Isolation and Purification of Ginsenoside Rb₁

Ginsenoside Rb_1 was isolated and purified from the crude saponin fraction of the rhizome of Panax Ginseng CA MEYER, Korea Red Ginseng, by repeated-column chromatography on silica gel with $CHCl_3$–MeOH–H_2O (65:35:10) and on octadecylsilyl (ODS) silica with MeOH–H_2O (1:1–7:3) (Samukawa et al, 1995).

Middle Cerebral Artery Occlusion in Spontaneously Hypertensive Rats-Stroke Prone

SHR-SP aged 16 to 18 weeks were used. Rats were anesthetized with 1.5% halothane in a 4:3 mixture of nitrous oxide and oxygen, and brain temperature was kept at 37.0°C±0.2°C during surgery. The left MCA above the rhinal fissure and distal to the striate branches was coagulated and cut (Zhang et al, 2004). An osmotic minipump (Model 2004 or 2001, Alza Corp., Palo Alto, CA, USA) filled with either gRb_1 solution or vehicle (saline) was implanted subcutaneously in the back of each animal before MCAO, and a fine silicon tube connected to the minipump was inserted into the left femoral vein after MCAO. After recovery from anesthesia, the animals were maintained in an air-conditioned room at approximately 22°C.

Measurement of Brain Temperature and Blood Pressure

The brain temperature of rats with or without gRb1 treatment was monitored by the combination of a temperature probe (XM-FH-BP 5 mm; Mini-Mitter Co., Sunriver, OR, USA) inserted into the right cerebral hemisphere (1 mm anterior to the bregma, 1.5 mm lateral to the midline) and the telemetry system receiving signals from the probe (DataScience Int., St Paul, MN, USA). The rats had free access to food and water except for the periods of minipump implantation and MCA occlusion. The brain temperature was continuously monitored until the end of the experiment.

The mean arterial blood pressure (MABP) in each animal was measured using a rat tail manometer–tachometer system (MK-1030, Muromachi Co., Tokyo, Japan).

Intravenous Infusion of Ginsenoside Rb₁ after Middle Cerebral Artery Occlusion in Spontaneously Hypertensive Rats-Stroke Prone

Ginsenoside Rb_1 was dissolved in isotonic saline. Then, 60 μL of gRb_1 solution (6 or 60 μg/60 μL) or the same volume of vehicle (saline) was injected into the left femoral vein of rats weighing 250 to 300 g immediately after MCAO, and then gRb_1 at a corresponding dose (6 or 60 μg/day, respectively) was continuously infused for 4 weeks through the minipump ($n=5$ to 8 per group). Middle cerebral artery-occluded animals infused with saline ($n=8$) were used as a control. Mean arterial blood pressure and the brain temperature of SHR-SP before MCAO were 203.1±6.9 mm Hg and 37.0°C±0.2°C, respectively. These were not affected by MCAO or gRb_1 infusion (data not shown).

Water Maze Test

The rats were subjected to repeated Morris water maze tests at 2 and 4 weeks after MCAO or sham operation. Each test included three trials per day for 4 consecutive days. The rats were allowed to swim until they reached the

submerged platform and to stay there for at least 10 secs. In the case that rats could not escape onto the platform within 90 secs, they were placed by hand onto the platform for 15 secs and their escape latency was recorded as 90 secs. The mean latency of finding the invisible platform was measured for individual animals on each day.

Morphologic Study

After water maze tests, the animals were anesthetized with an intraperitoneal injection of chloral hydrate (300 mg/kg) and perfused transcardially with 0.1 mol/L phosphate-buffered saline (pH 7.4), followed by perfusion with 500 mL of 4% paraformaldehyde in 0.1 mol/L phosphate buffer (pH 7.4). The brain was dissected out and fixed in the same fixative. After taking a photograph of each brain, the infarcted region was traced on a sheet. Then the areas of the infarcted region and the left hemisphere of the brain were measured by a computerized processing system. The ratio of the infarcted area to the left hemispheric area was calculated. The brain was embedded in paraffin, and serial coronal sections 5-μm thick were cut and stained with 0.1% cresyl violet.

The area of the thalamus was measured with a planimeter in sections 3.6 mm posterior to the bregma, and the ratio of the thalamic area on the MCA-occluded side to that on the contralateral side was calculated. Neuronal density per 0.099 mm² in the ventroposterior (VP) thalamic nucleus was evaluated in each animal. The ratio of the thalamic area and neuronal density in the VP thalamic nucleus were evaluated by an investigator without any prior information.

To investigate whether cerebrovascular networks were reorganized in the putative ischemic penumbra, paraffin sections from gRb₁-treated brain around the level 2.8 mm posterior to the bregma were examined with Nomarski optics ($n = 5$ per group). Four photomicrographs were taken from the parietal cortex of each cerebral hemisphere, and the vascular area per 1.27 mm² in the rescued parietal cortex on the lesioned side was compared with that in the counterpart on the contralateral side. Blood vessels identified in the photomicrographs were traced with black ink, and the vascular area was measured using NIH image software.

Delayed Intravenous Infusion of Ginsenoside Rb₁ after Middle Cerebral Artery Occlusion in Spontaneously Hypertensive Rats-Stroke Prone

Ginsenoside Rb₁ was dissolved in isotonic saline. Then, 60 μL gRb₁ solution (6, 60, 3000 or 12,000 μg/60 μL) or the same volume of vehicle (saline) was injected into the left femoral vein of rats weighing approximately 300 g at 2 h after MCAO, and then gRb₁ at a corresponding dose (6, 60, 3000 or 12,000 μg/day, respectively) was continuously infused through the minipump ($n = 7$ per group). The dose of continuous gRb₁ infusion was calculated as 20, 200 μg/kg per day, 10 or 40 mg/kg per day, respectively. Middle cerebral artery-occluded animals infused with saline ($n = 7$) were used as a control. At 1 day after MCAO,

animals were killed with an overdose of pentobarbital and decapitated. The brain was removed and sectioned coronally into seven 2-mm slices. Then the brain slices were subjected to 2,3,5-triphenyltetrazolium chloride (TTC) staining as described below.

2,3,5-Triphenyltetrazolium Chloride Staining

The brain slices were immediately stained with 2% TTC (Sigma, St Louis, MO, USA) and incubated at 37°C for 30 mins. The border between infarcted and noninfarcted tissue was outlined with an image analysis system, and the area of infarction was measured by subtracting the area of the nonlesioned ipsilateral hemisphere from that of the contralateral hemisphere (Swanson et al, 1990). The volume of infarction was calculated by integration of the lesion areas at all equidistant levels of the forebrain.

Three-Min Ischemia in Gerbils

Male Mongolian gerbils weighing 70 to 80 g (approximately 12 weeks of age) were used. The animals were anesthetized with 1.5% halothane in a 4:3 mixture of nitrous oxide and oxygen and placed in a stereotaxic apparatus. An osmotic minipump (Alza model 2001) filled with gRb₁ solution or vehicle (saline) alone was implanted subcutaneously in the back of each animal, and a needle connected to the minipump was placed in the left lateral ventricle at a point 1.5 mm anterior, 1.0 mm lateral and 2.7 mm ventral to the bregma. Three-minute occlusion of both common carotid arteries was performed as described previously (Morita et al, 2001).

Intracerebroventricular Infusion of Ginsenoside Rb₁ in Gerbils

Immediately after 3-min forebrain ischemia, 2 μL saline containing 2.5 or 25 ng gRb₁ was injected into the left lateral ventricle through a Hamilton syringe, and then gRb₁ (60 or 600 ng/day) was continuously infused for 7 days into the left lateral ventricle ($n = 8$ in each group). Control gerbils received the same volume of saline infusion after 3-min forebrain ischemia ($n = 6$).

In situ Detection of DNA Fragmentation (Terminal Deoxynucleotidyl Transferase-Mediated Deoxyuridine Triphosphate Nick End-Labeling (TUNEL) Staining)

After 3-min transient forebrain ischemia, gerbils were infused with 0, 60 or 600 ng/day gRb₁ ($n = 6$ to 8 in each group). At 7 days after the ischemic insult, animals were perfused with 4% paraformaldehyde in 0.1 mol/L phosphate buffer (pH 7.4) under pentobarbital anesthesia. Paraffin sections of the brain from each animal were processed for TUNEL staining (ApopTag, Intergen, NY, USA). All TUNEL-positive neurons along a 1-mm linear length of the CA1 field in two serial coronal sections were counted, and the mean number of positive neurons was calculated in each animal.

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Cortical Neuron Cultures

The cerebral cortices of 17-day-old rat embryos were aseptically dissected out. Cortical neurons were dissociated from the tissues as described elsewhere (Toku et al, 1998). The dissociated cells were seeded on 24-well plastic plates (Corning, New York, NY, USA) coated with poly-L-lysine, at a density of 2.5×10^5 cells/cm². The purity of the cultured neuron was confirmed by Western blot analysis with the antibodies against MAP2, GFAP and ED1 (Sugishita et al, 2001).

Sodium Nitroprusside (SNP) Treatment of Cultured Cortical Neurons

To induce apoptotic cell death, rat cortical neurons were exposed to an NO donor, 100 μmol/L SNP (Wako, Osaka, Japan), for 10 mins (Toku et al, 1998). Cortical neurons were cultured for 3 or 4 days in vitro, and then further cultured in the presence of 0 to 10^5 fg/ml gRb₁ for 24 h, followed by SNP treatment. After treatment, neurons were cultured for 16 h with 0 to 10^5 fg/ml gRb₁. Such low concentrations of gRb₁ were chosen in this experimental design on the basis of repeated preliminary studies with a wide range of gRb₁ concentrations.

Alamar Blue Assay

To evaluate the neuroprotective effect of gRb₁, survival of cultured cortical neurons was assessed, using Alamar blue (Alamar Biosciences, Sacramento, CA, USA) as a redox indicator (Morita et al, 2001).

Lipid Peroxidation Assay

Cortical neurons of 17-day-old rat embryos were cultured at a density of 8.0×10^5 cells/cm². On the fourth day of culture, a freshly prepared solution containing 100 nmol/L FeSO₄ and 1 mmol/L ascorbic acid was added to the medium, DMEM, to which 0.1 to 1000 fg/ml gRb₁ had been added on the second day of culture, and the neurons were maintained for 2 h at 37°C. Then, the relative level of lipid peroxidation in the cultured neurons was quantified by using 2-thiobarbituric acid fixation and subsequent measurement of light absorbance at 535 nm (Peng et al, 1998). Thiobarbituric acid reactive substances (TBARS) levels were expressed as malonyldialdehyde (MDA) equivalent (nmol/mL).

1,1-Diphenyl-2-picrylhydrazyl (DPPH) Radical Scavenging Assay

The activity of gRb1 to scavenge stable DPPH (Sigma Chemical Co., St Louis, MO, USA) radical was measured as described elsewhere (Mellors and Tappel, 1966). Briefly, DPPH radical was dissolved in ethanol to a final concentration of 500 μmol/L. Reactions were initiated by adding 100 μL of the freshly prepared DPPH radical solution to 100 μL gRb1 solution at various concentrations (0–100 mg/mL). The reactions were incubated at room temperature for 25 mins, and the absorbance at 517 nm was measured using a plate reader (model 550, Bio-rad Lab., Hercules, CA, USA).

Construction of Bcl-X Promoter Plasmids

The murine Bcl-X promoter fragment between positions −638 and −95 relative to the translation start site was prepared by polymerase chain reaction (PCR) with primers 5′-CCACGAGCTCGATCTGGTCGATGGAG GAAC-3′ and 5′-AAACACCTGCTCACTTACTGGGTC-3′. This segment, which contains the Stat5 response element (STRE), was digested by SacI and BamHI, and inserted between the SacI and BamHI sites of the pGL2-basic vector (Promega, Madison, WI, USA) to make the bcl-x-wt plasmid. A mutation in STRE was generated by PCR using a sense primer (5′-AGGCATTGAGGATAAAAGGG-3′) and an antisense primer (5′-CCCTTTTATCCTCAATGCCT-3′) carrying mutations, to make the bcl-x-mut plasmid. Another promoter fragment between positions −1217 and −592, which does not contain STRE, was also prepared by PCR with primers 5-CGGCCCTCGAGCCCTG CAGGGGGCT-3 and 5-AATTGCGAAGCTTAGGAACCTG CC-3, and inserted between the XhoI and HindIII sites of the pGL2-basic vector to make the bcl-x-0.6L plasmid (Silva et al, 1999).

Transient Transfection and Promoter Assays

Primary cultured neurons were seeded on 24-well PLL-coated plastic plates. On the third day of culture, the cells were cotransfected with 0.4 μg reporter plasmid and 0.1 μg pRL-TK (Promega) internal control plasmid using Lipofectamine Plus Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. After transfection, the cells were incubated at 37°C for 16 h. On the next day, gRb₁ was added to the medium at a concentration of 100 fg/mL, and neurons were incubated for a further 24 h. The activities of firefly luciferase from bcl-x promoter-luciferase plasmids and renilla luciferase from pRL-TK plasmid in the cell extracts were evaluated using a Dual-luciferase assay kit (Promega) with a luminometer (TD-20/20; Turner Designs Inc., Sunnyvale, CA, USA) according to the manufacturer's protocol.

Analysis of bcl-x_L mRNA Expression

Total RNA was extracted using Isogen (Nippon Gene, Tokyo, Japan), and treated with DNase. Oligo dT primers together with total RNA and Moloney murine leukemia virus reverse transcriptase (GibcoBRL) were used to obtain single-strand DNA. Polymerase chain reaction was conducted using Takara Taq polymerase (Takara, Tokyo, Japan). The following conditions were used for PCR amplification: cDNA products of the reverse transcription reaction were denatured for 2 mins at 94°C before 20 cycles (for β-actin) or 25 cycles (for bcl-x_L) at 94°C for 1.5 mins, 55°C for 1.5 mins and at 72°C for 2 mins. The reverse transcriptase-polymerase chain reaction (RT-PCR) products were separated on 3% agarose gel and visualized with ethidium bromide. The following pairs of oligonucleotides corresponding to certain sequences within the coding regions of the bcl-x_L and β-actin genes were used as primers: rat bcl-x_L primers, 5′ primer (5′-GTAGTGAATGA ACTCTTTCGGGAT-3′), 3′ primer (3′-CCAGCCGCCGTTCT

CCTGGATCCA-3′), rat β-actin primers, 5′ primer (5′-AGA AGAGCTATGAGCTGCCTGACG-3′) and 3′ primer (5′-TAC TTGCGCTCAGGAGGAGCAATG-3′). Polymerase chain reaction was performed five times for each sample.

Analysis of Bcl-x$_L$ Protein Expression

Homogenates were solubilized in Laemmli's sample solution containing 2% sodium dodecyl sulfate. An equal amount of protein (15 μg) from the homogenates was electrophoresed and processed for Bcl-x$_L$ immunoblot analysis using a monoclonal antibody against Bcl-x$_L$ protein (Transduction Laboratories Inc., Lexington, KY, USA). For semiquantitative evaluation, the immunoreactive bands were subjected to densitometric analysis.

Statistics

All values are presented as mean value $\pm$ s.d. Bcl-x$_L$ immunoreactivity in vehicle- and gRb$_1$-treated gerbils was analyzed statistically by two-tailed Mann–Whitney U-test. All other statistical significance was tested by one-way ANOVA followed by Bonferroni's multiple comparison test. A P-value less than 0.05 was considered to be statistically significant.

Results

Purity of Ginsenoside Rb$_1$

The purity of gRb$_1$ used in this study was more than 99.999%, as determined by high-performance liquid chromatography (Figure 1). Approximately 5 g of gRb1 was isolated and purified from 10 kg of Korea Red Ginseng, 6-year root.

Intravenous Infusion of Ginsenoside Rb$_1$ Ameliorates Cortical Infarct Size, Place Navigation Disability and Secondary Thalamic Degeneration in Middle Cerebral Artery-Occluded Spontaneously Hypertensive Rats-Stroke Prone

We first investigated the effects of postischemic intravenous infusion of gRb$_1$ on cortical infarct size, place-navigation disability and secondary thalamic degeneration in SHR-SP, with permanent occlusion of the left MCA above the rhinal fissure and distal to the striate branches. This MCA-occluded rat model has more severe neuronal damage than the gerbil forebrain ischemia model, and more closely mimics clinical infarction than do gerbils with transient forebrain ischemia (Coyle and Jokelainen, 1983; Igase et al, 1999; Tamura et al, 1981). All animals survived until the end of the experiment and exhibited cerebral infarction. The left cerebral hemisphere of MCA-occluded rats treated with vehicle (saline) alone for 28 days exhibited conspicuous cortical infarction (Figure 2A). When 6 or 60 μg/day gRb$_1$ was intravenously infused after permanent MCAO for 28 days, the infarct was markedly reduced in size (Figure 2B). It is possible that intravenous infusion of gRb$_1$ rescued many neurons in the ischemic penumbra. The proportion of infarct size in the group treated with gRb$_1$ (6 or 60 μg/day) was significantly smaller than that in the vehicle-treated group (Figure 2C). Nissl staining of saline-infused brain showed shrinkage and loss of neurons in the VP thalamic nucleus in comparison with thalamic neurons in sham-operated animals, whereas in gRb$_1$-treated ischemic brain, the majority of thalamic neurons appeared to be intact (Figures 2D to 2F). There was a significant difference in neuron number in the VP thalamic nucleus between the saline-infused ischemic group and the gRb$_1$-treated ischemic group (Table 1). As shown in Figure 2G and 2H, postischemic intravenous infusion of gRb$_1$ at a dose of 6 or 60 μg/day significantly decreased the escape latency on repeated trials of the Morris water maze test, especially on the fourth trial day at 2 weeks after MCAO and on the first, third and fourth trial days at 4 weeks after MCAO. There was no significant difference in swimming speed among the experimental groups (data not shown). Moreover, intravenous gRb$_1$ infusion did not affect cerebral blood flow, as monitored by laser Doppler flowmetry (Omega flow FLO-N1, Neuroscience Inc., Tokyo, Japan) (data not shown). These findings suggest that intravenous infusion of gRb$_1$ after permanent MCAO prevents place navigation disability, cortical infarction and secondary thalamic degeneration in SHR-SP. Surprisingly,
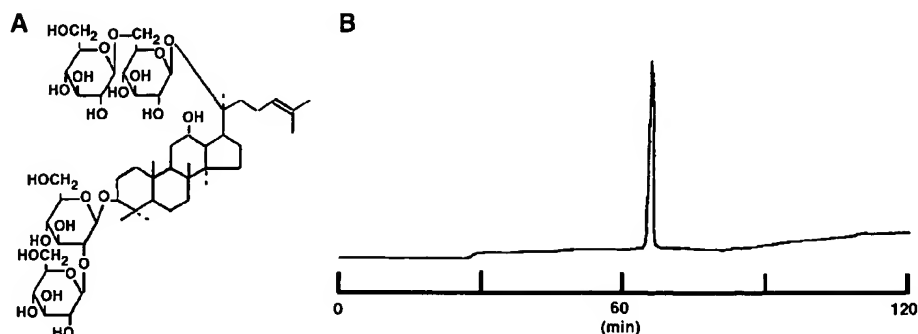

Figure 1 (A) Chemical structure of gRb$_1$. (B) HPLC purification of gRb$_1$. For details, see text.
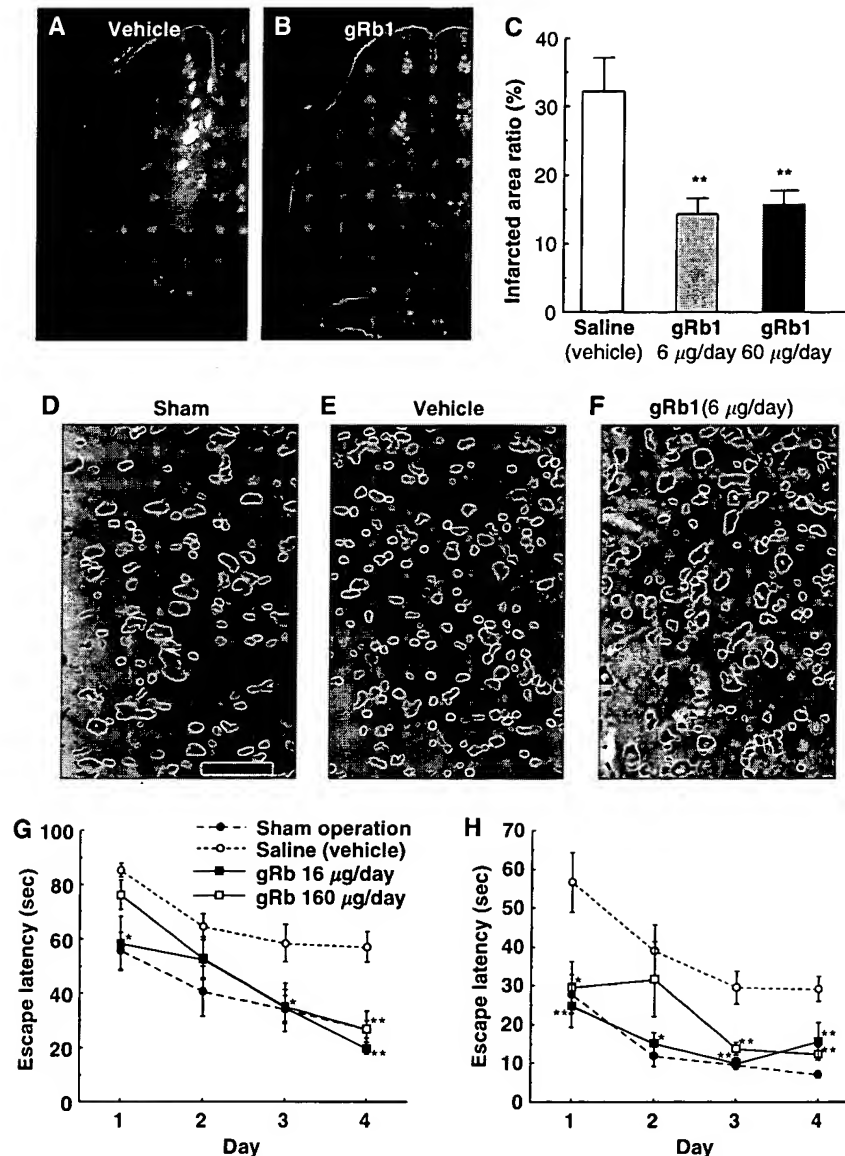
Figure 2 Intravenous infusion of gRb$_1$ ameliorates place navigation disability, cortical infarct size, and secondary thalamic degeneration in SHR-SP with permanent MCAO. (**A**) Photograph showing a cortical infarct in a vehicle-infused rat with permanent MCAO. (**B**) Photograph showing the cortical infarct in a gRb$_1$ (6 μg/day)-infused rat with permanent MCAO. (**C**) Effect of postischemic intravenous gRb$_1$ infusion on the ratio of the infarcted area to the left hemispheric area (n = 5–8, per group). (**D**) Nissl-stained section from a sham-operated rat showing intact VP thalamic neurons. (**E**) Nissl-stained section ipsilateral to the ischemic lesion, showing marked retrograde degeneration of VP thalamic neurons in a vehicle (saline)-infused ischemic brain. (**F**) Nissl-stained section showing that retrograde degeneration is prevented by intravenous infusion of gRb$_1$ (60 μg/day). Bar = 100 μm. (**G, H**) Morris water maze tests performed at 2 weeks (**G**) and 4 weeks (**H**) after MCAO in SHR-SP that received intravenous infusion of either vehicle (saline) or gRb$_1$ (6 or 60 μg/day) after focal cerebral ischemia (n = 5–8 per group).

the neuroprotective effect of intravenously infused gRb$_1$ at a dose of 6 μg/day was much more potent than that of intracerebroventricularly infused gRb$_1$ at the same dose, and it was as potent as the neuroprotective effect of intracerebroventricularly infused gRb$_1$ at a dose of 0.6 μg/day. Moreover, intravenous infusion of gRb$_1$ ameliorated ischemia-induced neuronal damage over a wider dose range than intracerebroventricular infusion of this agent (Zhang et al, 1998). The neuroprotective effects of gRb$_1$ appeared

to be ascribable, at least in part, to prevention of neuronal apoptosis, which is known to occur in the ischemic penumbra (Ferrer and Planas, 2003).

Intravenous Infusion of Ginsenoside Rb$_1$ Results in Reorganization of Disrupted Cerebrovascular Networks in Ischemic Penumbra

We next investigated whether the cerebrovascular networks disrupted by permanent MCAO were

Table 1 Left-to-right ratio of thalamic area and VP thalamic neuron number

	N	Ratio of area (%)	VP neuron number
Vehicle	8	86.4±8.1	10.1±5.5
gRb₁: 6 μg/day	5	95.9±6.6	30.6±5.4**
gRb₁: 60 μg/day	8	95.3±7.2	31.5±9.6**
Sham	8	98.8±5.3*	58.5±4.7**

Data are represented as means±s.d.
*$P < 0.05$; **$P < 0.01$ versus Vehicle.

reorganized in the putative ischemic penumbra, which was rescued by intravenous infusion of gRb₁. Paraffin sections from gRb₁-treated brains around the level 2.8 mm posterior to the bregma were examined with Nomarski optics (Figure 3A), and vascular (blood vessel) area per 1.27 mm² in the rescued parietal cortex 300 μm away from the site of marked gliosis in the periphery of the cortical infarct was compared with that in the counterpart on the nonlesioned (contralateral) side (Figures 3B and 3C). As shown in Table 2, there was no significant difference in vascular area between the sides. Although clear demarcation of penumbra was not defined in this study, it is likely that 28-day intravenous infusion of gRb₁ after permanent MCAO resulted in almost complete reorganization of cerebrovascular networks in the putative ischemic penumbra rescued. For these reasons, treatment of cerebral infarction with intravenous infusion of gRb₁ appeared to be complete by 28 days after permanent MCAO, and, hopefully, termination of gRb₁ treatment at this time would no longer lead to aggravation of neuronal damage in the MCA-occluded brain.

Assessment of Therapeutic Time Window for Neuroprotective Effect of Ginsenoside Rb₁

After confirming the reorganization of cerebrovascular networks, we investigated the effect of gRb₁ in the situation where its first administration was delayed for 2 h after MCAO. As shown in Figure 4, a significant reduction in total infarct volume was observed when the treatment was delayed until 2 h after MCAO and gRb₁ was administered at low doses (6 or 60 μg/day). However, this protective effect of gRb₁ was not evident when administered at high doses (3 or 12 mg/day). In addition, as shown in Table 3, there were no significant differences in MABP and brain temperature among three groups (0, 6 μg/day, 12 mg/day).

Ginsenoside Rb₁ Prevents Apoptotic Cell Death of Neurons In Vitro

To confirm the antiapoptotic action of gRb₁, we investigated whether gRb₁ prevented neuronal apoptosis, which was induced by 10-min exposure of
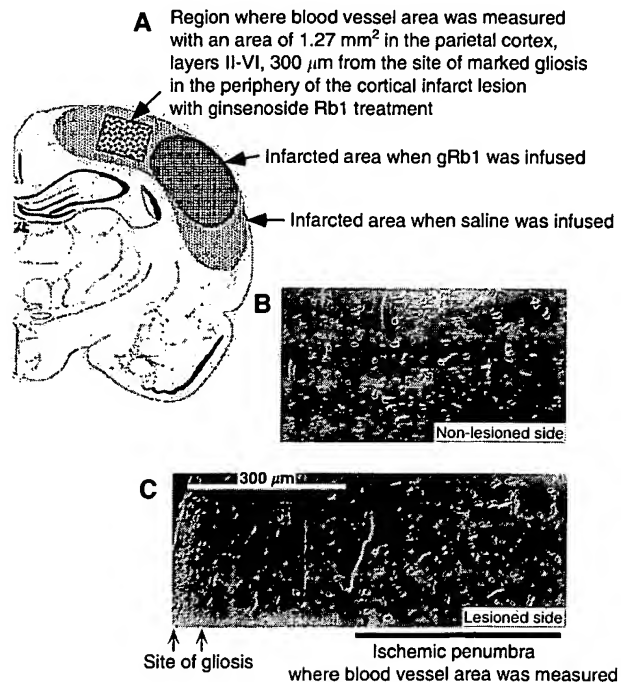

Figure 3 Cerebrovascular networks disrupted by permanent MCAO are reorganized in the putative ischemic penumbra, which was rescued by intravenous infusion of gRb₁. (A) Schematic representation showing the site for measurement of cerebrovascular area in the putative ischemic penumbra (layers II–IV of the parietal cortex) rescued by intravenous infusion of gRb₁. (B, C) Photomicrographs taken with Nomarski optics, showing cerebrovascular networks in the parietal cortex on the nonlesioned (control) side (B) and in the parietal cortex on the lesioned (ischemic) side (C). Note that the cortical region approximately 300 μm away from the site of marked gliosis in the periphery of the cortical infarct contains blood vessels comparable to those in the counterpart on the nonlesioned side. Regions that were presumably rescued by intravenous infusion of gRb₁ were chosen for the measurement of cerebrovascular area.

Table 2 Ratio of cerebrovascular area to 1.27 mm² brain area

	N	Contralateral side (%)	Lesioned side (%)
gRb₁: 6 μg/day	5	7.0±0.64	8.0±0.58
gRb₁: 60 μg/day	5	8.3±0.92	9.0±0.52

Ratio of cerebrovascular area to 1.27 mm² brain area in the ischemic penumbra on the lesioned side rescued by intravenous infusion of ginsenoside Rb₁ and in the counterpart on the contralateral side.
Data are represented as means±s.d.

cultured neurons to the NO donor SNP, using alamar blue assay (Toku et al, 1998). Ginsenoside Rb₁ at concentrations of 1 to 10⁵ fg/mL did not affect the viability of control cultured cortical neurons without SNP treatment (Figure 5A). When cortical neurons were exposed to 100 μmol/L SNP for 10 mins, many of them underwent apoptosis within 16 h (Figure 5B), as described by Toku et al (1998). However, when cortical neurons were incubated

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with 1 or 100 fg/mL gRb₁ before, during and after SNP treatment (indicated as 'Through' in Figure 5B), cell viability was maintained at a level close to that of control cortical neurons not exposed to SNP (Figure 5A). Furthermore, 1 or 100 fg/mL gRb₁ was similarly effective in rescuing cortical neurons when added to neuronal cultures only after SNP treatment (indicated as 'Post' in Figure 5B). The antiapoptotic action of gRb₁, which was added to the cultured neurons after SNP treatment, implies that gRb₁ activates intracellular signaling to promote neuronal survival rather than acting as a scavenger of NO *per se*. This speculation was further reinforced by the finding that gRb₁ at concentrations of 1 to 1000 fg/mL did not significantly attenuate membrane lipid peroxidation induced by the hydroxyl-radical-promoting agent (FeSO₄), although all values of TBARS were significantly increased after FeSO₄
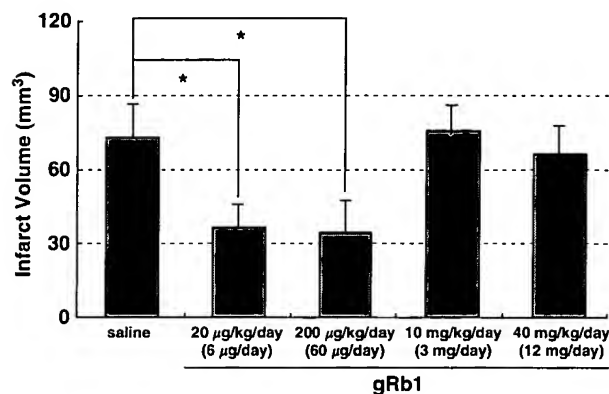

Figure 4 Effect of gRb₁ administered at 2 h after permanent MCAO on total infarct volume in SHR-SP. Vehicle or gRb₁ was administered at a predetermined dose (6, 60, 3000 or 12,000 μg/day). Note that gRb₁ at doses of 6 and 60 μg/day significantly reduced the total infarct volume. *N* = 7 per group.

treatment (Figure 5C). Furthermore, as shown in Table 4, gRb₁ did not exhibit DPPH radical scavenging activity at concentrations lower than 100 mg/mL. Thus, gRb₁ added to cultured neurons either before or after SNP treatment prevents neurons from undergoing apoptosis, and the present *in vitro* experiments do not support the previous notion that gRb₁ protects ischemic neurons by suppressing oxygen-free radical-induced lipid peroxidation (Lim *et al*, 1997).

Ginsenoside Rb₁ Upregulates Expression of Antiapoptotic Factor Bcl-x$_L$ In Vitro

To gain an insight into the molecular mechanisms underlying gRb₁-mediated neuronal survival, we investigated gRb₁-induced changes in the expression of Bcl-2, Bcl-w, Bcl-x$_L$, Bcl-X$_S$, apoptosis activating factor-1 (Apaf1), Akt, Bax, Bak, Bad, Fas, Fas ligand, P53, P21 and GADD45 mRNA in neurons, using RT-PCR. Among the apoptosis-regulating factors examined, Bcl-x$_L$ mRNA expression was markedly upregulated by treatment with gRb₁ at concentrations of 1 to 100 fg/mL, which were the optimal concentrations for gRb₁ to exhibit an antiapoptotic action. Reverse transcriptase-polymerase chain reaction using specific primers that amplify a 189-bp fragment of rat Bcl-x$_L$ mRNA detected a PCR product of the expected size, and its identity was confirmed by direct sequencing. As shown in Figure 5D, cortical neurons treated for 24 h with 1 or 100 fg/mL gRb₁ showed an increase in Bcl-x$_L$ mRNA expression compared with the gene expression of control cortical neurons. Densitometric analysis showed that neurons cultured in the presence of 1 or 100 fg/mL gRb₁ expressed approximately 6 times as much Bcl-x$_L$ mRNA as that expressed in neurons without gRb₁ treatment. Neurons cultured with 10⁵ fg/mL gRb₁ did not

Table 3 Physiological parameters

gRb₁	Vehicle control group	Low-dose group (6 μg/day)	High-dose group (12 mg/day)
Mean arterial blood pressure (mmHg)			
Before MCA	204.0±6.5	184.3±14.2	197.3±3.5
0 h after MCAO	199.3±6.4	180.3±22.2	191.8±12.8
2 h after MCAO (before infusion)	187.5±4.8	189.8±10.3	179.5±9.8
4 h after MCAO	183.0±9.4	197.8±14.5	184.8±6.8
12 h after MCAO	195.8±6.8	206.3±9.6	182.8±12.3
24 h after MCAO	185.8±12.0	195.8±16.6	185.0±6.4
Brain temperature (°C)			
Before MCAO	37.0±0.2	37.0±0.2	37.0±0.3
0 h after MCAO	36.8±1.1	36.8±0.2	36.9±0.5
2 h after MCAO (before infusion)	36.7±0.8	36.7±0.5	37.4±0.6
4 h after MCAO	37.1±0.7	36.9±0.4	37.5±0.7
12 h after MCAO	37.6±0.4	37.4±0.5	37.6±0.6
24 h after MCAO	37.8±0.6	37.2±0.3	37.7±0.6

gRb₁: ginsenoside Rb₁.
MCAO: middle cerebral artery occlusion.
Data are represented as means±s.d. *n* = 5 in each group.
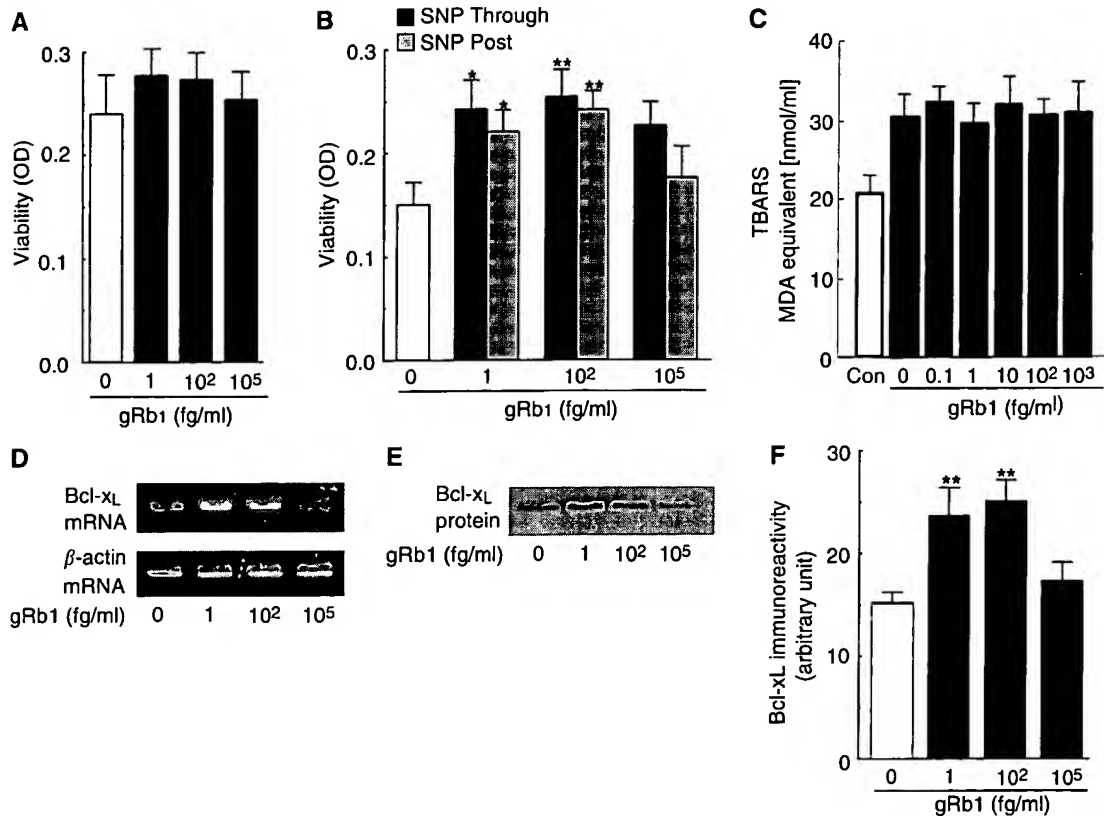There are no significant differences among three groups.

Figure 5 Prevention of NO-induced neuronal apoptosis by gRb₁ through upregulation of Bcl-x_L expression. (**A**) Effect of 0 to 10^5 fg/mL gRb₁ on viability of control cultured cortical neurons not exposed to SNP. (**B**) Viability of cultured cortical neurons incubated with 0 to 10^5 fg/mL gRb₁ before, during and after ('Through', solid bars) or only after ('Post', hatched bars) 100 μmol/L SNP treatment. (**C**) Effect of gRb₁ on membrane lipid peroxidation induced by $FeSO_4$. (**D**) Semiquantitative RT-PCR analysis of Bcl-x_L mRNA in cortical neurons cultured for 24 h in the presence of gRb₁. (**E**) Immunoblot analysis of Bcl-x_L protein expression in cultured cortical neurons. Ginsenoside Rb₁ at concentrations of 1 and 100 fg/mL clearly induced Bcl-x_L protein expression in cultured neurons. (**F**) Densitometric analysis of Bcl-x_L-immunoreactive bands, showing a 50% increase in Bcl-x_L protein expression in cortical neurons treated for 48 h with 1 or 100 fg/mL gRb₁. Data were obtained from five independent experiments.

Table 4 Effects of ginsenoside Rb₁ (gRb₁) on DPPH radical

gRb₁	Absorbance (% of control)
Vehicle control	100.0 ± 1.6
0.1 fg/mL	99.9 ± 1.1
1 fg/mL	98.6 ± 1.9
10 fg/mL	100.3 ± 1.9
100 fg/mL	100.1 ± 1.7
1 pg/mL	98.6 ± 2.5
100 ng/mL	98.8 ± 1.3
1 mg/mL	98.8 ± 2.0
10 mg/mL	97.9 ± 1.8
100 mg/mL	$91.5 \pm 0.4^{**}$

Data are represented as means $\pm$ s.d. $n = 6$ in each group.
$^{**}P < 0.01$ versus Vehicle control.

exhibit any detectable change in Bcl-x_L mRNA expression. In addition, we conducted Western blot using an antibody against Bcl-x_L protein. Bcl-x_L protein with a molecular mass of approximately 29 kDa was constitutively expressed in cultured

neurons (Figure 5E). Consistent with the increase in Bcl-x_L mRNA expression, Bcl-x_L protein expression was clearly increased in neurons cultured for 48 h in the presence of 1 or 100 fg/mL gRb₁ (Figure 5E). Densitometric analysis of the immunoreactive bands revealed that gRb₁ treatment caused a 50% increase in Bcl-x_L protein expression in cortical neuron cultures (Figure 5F). These findings suggest that gRb₁ prevents neuronal apoptosis through upregulation of the antiapoptotic factor, Bcl-x_L. To our knowledge, gRb₁ is the only nonpeptide agent that facilitates neuron survival by upregulating Bcl-x_L protein expression.

Induction of Bcl-x_L mRNA by Ginsenoside Rb₁ is Stat5 Dependent

To determine whether induction of Bcl-x_L mRNA by gRb₁ is actually dependent on activation of the transcription factor signal transducer and activator

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of transcription 5 (Stat5), we constructed bcl-x promoter-luciferase plasmids carrying a wild-type STRE (bcl-x-wt) or a mutant STRE (bcl-x-mut), and a plasmid that does not carry STRE (bcl-x-0.6L) (Figure 6A). Primary cultured neurons were transfected with these promoter-reporter plasmids and incubated for 24 h in the presence or absence of gRb₁ (100 fg/mL). Then, luciferase activity in the cell lysate was assayed. Consequently, luciferase activity in gRb₁-treated neurons was significantly higher than that in untreated neurons when the cells were transfected with bcl-x-wt plasmid (2.7-fold; $P < 0.01$; Figure 6B). When bcl-x-mut or bcl-x-0.6L plasmid was used for transfection, there was no significant difference between gRb₁-treated neurons and untreated neurons (Figure 6B). These findings indicate that Stat5 responsive element on the bcl-x$_L$ promoter is responsible for the induction of bcl-x$_L$ mRNA by gRb₁ in cultured neurons.

Ginsenoside Rb₁ Prevents Delayed Neuronal Death and Induces Bcl-x$_L$ Protein *In Vivo*

We next attempted to confirm that gRb₁ prevents delayed neuronal death and upregulates Bcl-x$_L$ protein expression *in vivo*. We used a gerbil model with transient forebrain ischemia of 3-min duration. Gerbils are known to show a loss of nearly one half of hippocampal CA1 neurons 7 days after 3-min ischemia (Morita *et al*, 2001; Sakanaka *et al*, 1998), and, moreover, many of the surviving CA1 neurons in the same period exhibit TUNEL-positive reactions. We investigated whether intracerebroventricular gRb₁ infusion (60 or 600 ng/day), starting after 3-min forebrain ischemia, reduced the number of TUNEL-positive neurons in the hippocampal CA1 field. As described before (Morita *et al*, 2001; Wen *et al*, 1998), many TUNEL-positive neurons were present in the hippocampal CA1 field of 3-min ischemic gerbils with saline infusion 7 days after the ischemic insult (Figure 7A), suggesting that irreversible neuronal degeneration was in progress during this period. Intracerebroventricular infusion of gRb₁ caused a significant decrease in the number of TUNEL-positive neurons in a dose-dependent manner (Figures 7B to 7D). Moreover, the CA1 field of gRb₁ (600 ng/day)-infused gerbils, when investigated with Western blot, contained a larger amount of Bcl-x$_L$ protein than that of vehicle (saline)-treated gerbils at 2 days after 3-min forebrain ischemia (Figures 7E and 7F). These findings suggest that intracerebroventricular infusion of gRb₁ prevents delayed neuronal death *in vivo* through upregulation of Bcl-x$_L$ protein expression.

Intravenous Infusion of Ginsenoside Rb₁ Upregulates Bcl-x$_L$ mRNA Expression in Middle Cerebral Artery-Occluded Brain

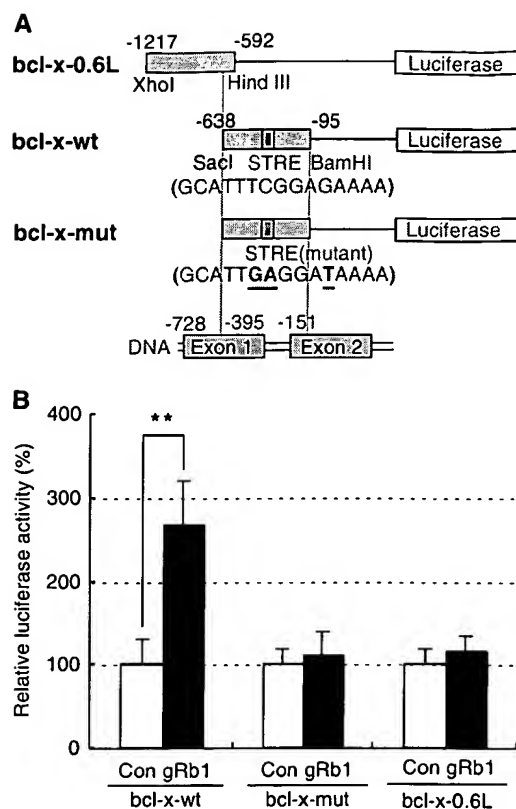To confirm that intravenous infusion of gRb₁ induces Bcl-x$_L$ expression in the ischemic brain,

Figure 6 Transcriptional activation of luciferase reporter construct driven by bcl-x promoter in response to gRb₁ treatment. **(A)** Schematic representation of bcl-x promoter region containing the fragment bcl-x-wt, bcl-x-mut or bcl-x-0.6L used in this study. **(B)** Cultured neurons were cotransfected with a renilla luciferase reporter vector (pRL-TK) and the following constructs: bcl-x-wt, which carries STRE, bcl-x-mut, in which the STRE has been mutated and bcl-x-0.6L, which does not carry the STRE. Neurons were incubated for 24 h in the presence or absence of gRb₁ (100 fg/mL). Units of luciferase activity were normalized based on values of pRL-TK activity to control for transfection efficiency. Data were obtained from five independent experiments.

we next investigated Bcl-x$_L$ mRNA expression within the cerebral cortex on the lesion side at 12 h after gRb₁ (6 or 60 μg/day) or vehicle (saline) infusion in MCA-occluded rats, using RT-PCR under semiquantitative conditions. The PCR product showed the expected size, and its identity was confirmed by direct sequencing. The lesioned cerebral cortex of MCA-occluded rats with intravenous infusion of gRb₁ exhibited higher Bcl-x$_L$ mRNA expression than that of rats with vehicle infusion (Figure 8A). As shown in Figure 8B, Bcl-x$_L$ mRNA expression in the gRb₁-treated group was significantly (approximately 3 times) higher than that in the untreated group. These data clearly show that intravenous infusion of gRb₁ upregulated Bcl-x$_L$ mRNA expression in the MCA-occluded rat brain.
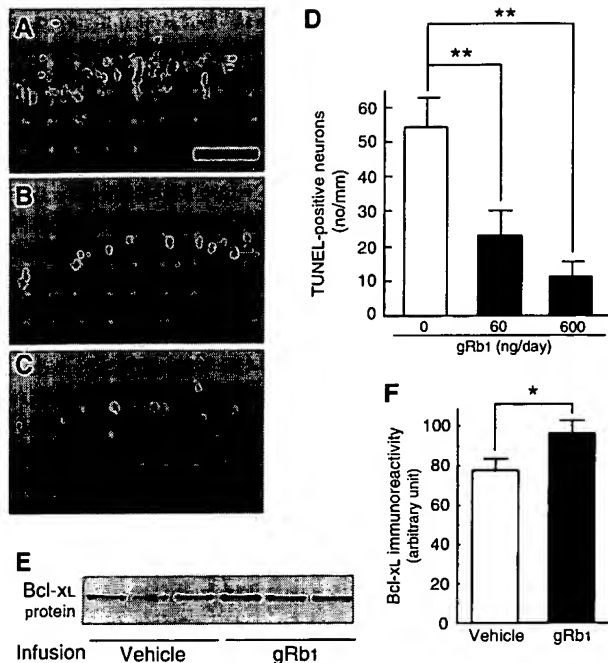
Figure 7 Intracerebroventricular infusion of gRb₁ prevents apoptotic neuron death in hippocampal CA1 field of gerbils with transient forebrain ischemia of 3-min duration. **(A to C)** Photomicrographs of TUNEL-positive neurons in the CA1 field of ischemic gerbils after 7-day infusion of vehicle or gRb₁. **(A)** Vehicle, **(B)** 60 ng/day gRb₁, **(C)** 600 ng/day gRb₁. Bar = 200 μm. **(D)** Number of TUNEL-positive neurons in the hippocampal CA1 field of ischemic gerbils ($n = 8$ per group). Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling-positive neurons in the hippocampal CA1 field in gRb₁ (60 or 600 ng/day)-infused ischemic gerbils were fewer than those in vehicle-infused ischemic animals. **(E)** Immunoblot analysis of Bcl-x_L protein in the CA1 field of ischemic gerbils infused with vehicle (saline) or gRb₁ (600 ng/day) at 2 days after 3-min ischemia. **(F)** Densitometric analysis of Bcl-x_L immunoreactive bands, showing a significant increase in Bcl-x_L protein in the CA1 field in gRb₁-infused ischemic gerbils.

Figure 8 Intravenous infusion of gRb₁ upregulates Bcl-x_L mRNA expression in the ischemic cortex of the MCA-occluded rat. **(A)** Semiquantitative RT-PCR analysis of Bcl-x_L mRNA in the ischemic cortex of MCA-occluded rats with the treatment of gRb₁ (0, 6, 60 μg/day) for 12 h. β-Actin was also amplified as an internal control from each sample. **(B)** Densitometric analysis showing an approximately 200% increase in Bcl-x_L mRNA expression in ischemic cortex treated with gRb₁ (6 or 60 μg/day). Data were obtained from five independent experiments.

Discussion

In the present study, we showed that gRb₁ upregulated the expression of Bcl-x_L, both *in vivo* and *in vitro*. Moreover, we revealed that a Stat5 responsive element in the bcl-x promoter became active in response to gRb₁. This was confirmed by the findings that mutagenesis or deletion of this sequence motif abrogated its promoter activity. Bcl-x_L protein can act upstream of mitochondria in the initiation of apoptosis and inhibit the mitochondrial cell death signaling pathway, suggesting that Bcl-x_L is situated in the center of neuron survival signaling via mitochondria. Therefore, we speculate that upregulation of Bcl-x_L protein by gRb₁ is a safer and more practical way of neuroprotection than caspase inhibition, if the mitochondrial cell death signaling pathway becomes irreversible after libera-
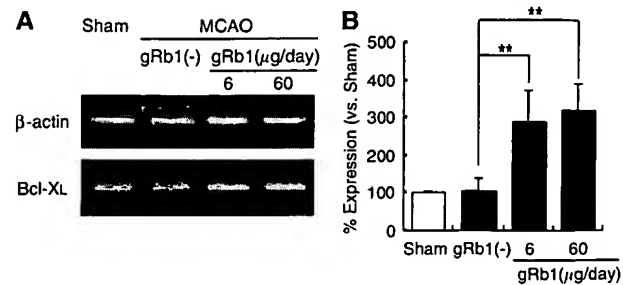
tion of Apaf1 from Bcl-x_L. In fact, our study revealed that gRb₁ rescued cortical neurons in the ischemic penumbra and reduced the volume of the cortical infarct by approximately 50%, without any noticeable adverse effects. In addition, we showed the reorganization of vascular networks in the ischemic penumbra after the treatment of gRb₁. Although clear demarcation of ischemic penumbra was not defined in this study, it is likely that the reorganization of vascular networks in the putative ischemic penumbra also plays an essential role in the effects of gRb₁ on neural injury. Further investigation must be required to clarify the neuroprotective machanism of gRb₁.

Previously, we showed that gRb₁ significantly prevents delayed neuronal death in the hippocampal CA1 field of the gerbil when injected intraperitoneally for 7 days before transient forebrain ischemia (Lim *et al*, 1997; Wen *et al*, 1996). However, intraperitoneal injections of gRb₁, starting immediately after transient forebrain ischemia, do not rescue hippocampal CA1 neurons from delayed neuronal death (Lim *et al*, 1997; Wen *et al*, 1996). Presumably, it takes a considerable time for intraperitoneally injected gRb₁ to reach the brain, and thus, postischemic gRb₁ injections into the peritoneal cavity cannot protect ischemic hippocampal CA1 neurons in the critical periods when neuronal death or survival is determined. In support of this speculation, intracerebroventricular infusion of gRb₁ starting immediately after transient forebrain ischemia rescued a significant number of hippocampal CA1 neurons from lethal damage (Lim *et al*, 1997). Using SHR-SP with permanent MCAO, we previously showed that intracerebroventricular gRb₁ infusion starting just after permanent MCAO ameliorates ischemia-induced place navigation disability and cortical infarction (Zhang *et al*, 1998). These findings suggest that centrally, but not peripherally,

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infused gRb₁ exerts a curative effect on ischemic neuronal damage. Before conducting the current experiments, we speculated that it is extremely difficult to apply gRb₁ for the treatment of cerebral infarction, because (1) the effective dose range of gRb₁ infused intracerebroventricularly after MCAO is quite narrow; only 0.6 μg/day (but neither 6 nor 0.06 μg/day) gRb₁ is effective in ameliorating cortical infarct size and place navigation disability in MCA-occluded rats, and (2) intracerebroventricular infusion of gRb₁ is not a practical way for drug delivery in clinical medicine. In the present study, we showed that postischemic intravenous infusion of gRb₁ ameliorates, in a wide dose range (6 -to 60 μg/day), place navigation disability and cortical infarct size in SHR-SP with permanent occlusion of the MCA distal to the striate branches. It is not always easy to state with certainty why intravenous but not intracerebroventricular infusion of gRb₁ exhibits a wide range of effective doses. A possible explanation for this is that the blood brain barrier regulates the central adsorption of gRb₁ from the blood stream, so that an optimal amount of gRb₁ is transported into the ischemic brain. Presumably, the high stability of gRb₁, having both hydrophilic and hydrophobic chemical structures, appears to enable such elegant regulation. Since gRb₁ has no site for hapten binding, it is difficult to raise antibodies against gRb₁. Moreover, labeling of gRb₁ with radioisotopes is not possible at this time, because it cannot be artificially synthesized. Nevertheless, a specific antibody with high sensitivity to gRb₁, if produced in the future, would clarify the central distribution and levels of intravenously infused gRb₁ in vivo.

To clarify the neuroprotective mechanisms of gRb₁, we focused on upregulation of Bcl-x_L gene expression by gRb₁, because (1) the Bcl-x_L gene product is a mitochondrion-associated protein that acts as a key molecule facilitating neuron survival (Polster and Fiskum, 2004); (2) it is abundant in mature neurons of the adult brain in which Bcl-2 expression declines to very low levels (Gonzalez-Garcia et al, 1995); (3) the expression of Bcl-x_L protein is markedly increased in neurons close to the ischemic penumbra (Isenmann et al, 1998; Wen et al, 1998); (4) the size of infarcts resulting from permanent occlusion of the MCA was not affected by overexpression of Bcl-2, but was reduced by overexpression of Bcl-x_L (Wiessner et al, 1999); and (5) IL-3 and erythropoietin, which have protective actions similar to those of gRb₁ on the ischemic hippocampus and on cultured neurons treated with FeSO₄, are known to rescue damaged neurons through upregulation of Bcl-x_L gene product (Wen et al, 2002, 1998).

Bcl-x_L protein is known to suppress activation of procaspase 9 by forming a complex with Apaf1 and to prevent the release of cytochrome c from mitochondria, thereby maintaining cell viability and cell survival. Bcl-x_L protein is also known to prevent the release of second mitochondrial activa-

tion of caspases (Smac)/direct inhibitor of apoptosis binding protein with low pI (DIABLO) from mitochondria. Second mitochondrial activation of caspases /DIABLO antagonizes X-linked inhibitor of apoptosis protein (XIAP), which blocks the second proteolytic step for full caspase-3 activation (Sprick and Walczak, 2004). Furthermore, the redistribution of apoptosis-inducing factor (AIF) from mitochondria to the nucleus is prevented by Bcl-x_L, leading to inhibition of cell death signaling through a caspase-independent pathway (Ferrer and Planas, 2003). In the ischemic hippocampus, mismatch between Bcl-x_L mRNA and protein expression is noted in the early postischemic period (Wen et al, 1998), and insufficient translation of Bcl-x_L protein in response to the ischemia-induced increase in Bcl-x_L mRNA appears to liberate Apaf1 and cytochrome c, which form a complex with procaspase 9, leading to activation of procaspase 9 and caspase 9, and then to activation of the cell executioner, caspase 3 (Love, 2003). Moreover, neuronal death after mild focal ischemia in mice has been shown to be alleviated by intracerebroventricular injection of caspase inhibitors (Endres et al, 1998). It has been reported that a novel small peptidomimetic oxoazepinoindoline caspase inhibitor that crosses the blood–brain barrier reduced the infarct size after both transient and permanent focal brain ischemia, even when administered intravenously (Deckwerth et al, 2001). These findings are in favor of the notion that the mitochondrial cell death signaling pathway is involved in the occurrence of ischemic neuronal death, particularly within the hippocampal CA1 field and ischemic penumbra. Although the most effective way to treat ischemic neuronal damage might be to suppress caspase activity in damaged neurons, the neuroprotective effects of caspase inhibitors have been verified only up to 7 days after mild focal ischemia in mice (Endres et al, 1998). In other words, it cannot be ascertained that the temporary suppression of caspase activity by intracerebroventricular infusion of caspase inhibitors eventually rescues ischemic neurons that are committed to die by caspase activation; the damaged neurons may undergo delayed degeneration as the caspase inhibitors are degraded. In fact, one note of caution comes from the observation that intracerebroventricular infusion of a caspase inhibitor prevented delayed loss of neurons from the CA1 field of the hippocampus after ischemia, but did not prevent impairment of induction of long-term potentiation (Gillardon et al, 1999). The implication is that interference with caspase could lead to the preservation of damaged, malfunctioning neurons.

There have been many in vitro studies dealing with the PI3K–Akt–Bad pathway affecting the antiapoptotic function of Bcl-x_L (Love, 2003). The level of activated Akt increased within hours after the onset of ischemia and subsequently declined (Noshita et al, 2001). Infarct volume after MCAO was significantly reduced by overexpression of the

active form of Akt under the control of the damage-induced neuronal endopeptidase (DINE) promoter (Ohba et al, 2004). However, phosphorylation of Bad was not detected after cerebral ischemia, despite an increase in the level of phosphorylated Akt (Friguls et al, 2001). The precise mechanism of the PI3K–Akt–Bad pathway after ischemia remains unclear. In addition to the mitochondrial cell death signaling pathway, the death receptor-mediated signaling pathway might also be involved in neuronal cell death after ischemia. One of the most investigated death receptors after cerebral ischemia is CD95/Fas. Although early studies showed that CD95/Fas was not expressed in the adult brain (Siesjo et al, 1999), recent studies showed that CD95/Fas was expressed in the brain after cerebral ischemia (Jin et al, 2001; Padosch et al, 2003). Infarct size resulting from focal cerebral ischemia is greatly reduced in lymphoproliferation mutant (lpr) mice expressing dysfunctional Fas (Martin-Villalba et al, 1999). At present, we do not know much about the interactions between gRb$_1$ and CD95/Fas or Akt. Thus, it is expected that further studies on the intracellular survival signals stimulated by the potent neuroprotectant, gRb$_1$, will lead to elucidation of the molecular mechanisms underlying ischemic neuronal death.

Another advantage of gRb$_1$ might be that it does not affect cell viability when added to control neuronal cultures, despite its potent antiapoptotic action on SNP-treated neurons. Furthermore, intracerebroventricularly infused gRb$_1$ has been shown to exert no effects on cerebral blood flow, brain temperature or MABP (Lim et al, 1997; Zhang et al, 1998), and intravenous gRb$_1$ infusion did not affect brain temperature or MABP in the present study. These findings suggest that gRb$_1$ rescues ischemic neurons without affecting physiological parameters or possibly neural transmission. Since ginseng has been taken by many people in Asian countries for thousands of years, gRb$_1$ as an ingredient of ginseng is expected to cause few adverse effects in humans. It is tempting to speculate that intravenous infusion of gRb$_1$ could be applied in patients with acute cerebral stoke in the ambulance before diagnosis of the type of stroke by computed tomography. Ginsenoside Rb$_1$, which upregulates Bcl-x$_L$ at extremely low concentrations, might also be used for the treatment of neurodegenerative diseases, ischemia–reperfusion injury, graft-versus-host disease and autoimmune disorders involving activation of mitochondrial cell death signaling.

In conclusion, the present experimental results introduce a new concept of gRb$_1$-mediated general cytoprotection to biomedical research. The precise intracellular mechanism(s) by which gRb$_1$ induces Bcl-x$_L$ protein production remains to be determined, but the present study seems to open a new horizon in research on intravenously infusible neuroprotective agents that may rescue millions of patients with diseases of the brain, generation after generation.

Acknowledgements

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